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Polypeptide components of human small nuclear ribonucleoproteins

(small nuclear RNAs/autoimmune antibodies/U1-specific polypeptide/phosphorylation)

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ABSTRACT **Small nuclear RNA molecules (snRNAs) are associated with polypeptides *in vivo*, forming small nuclear ribonucleoprotein complexes (snRNPs). These snRNP complexes are targets for certain autoimmune antisera. Antisera of the type anti-Sm precipitate (and therefore define) a class including U1, U2, U4, U5, and U6 snRNAs, whereas antisera of the anti-RNP type precipitate only U1 snRNPs. We used these two types of autoimmune antisera (from patients with systemic lupus erythematosus) to study the polypeptide components in human cells. Sequential immunoprecipitation of the complexes from nuclear extracts with anti-RNP and anti-Sm antibodies, along with radioimmunoassay of protein transfers, identified four polypeptides of 14,000 (P14), 17,000 (P17), 26,000 (P26), and 27,000 (P27) daltons that are present on all members of this class, whereas a 68,000-dalton (P68) polypeptide is present only on U1 snRNPs. Based on the radioimmunoassay, three of these polypeptides, P17, P26, and P27, are also the antigens for anti-Sm antisera, whereas P68 is the antigen for anti-RNP antisera. Long-term phosphate labeling experiments show that the only detectably phosphorylated polypeptide is P68, which contains phosphoserine.**

A number of discrete, small RNA species, ranging in size from about 80 to about 350 nucleotides, have been characterized over the past decade as ubiquitous components of eukaryotic cells (reviewed in ref. 1). Among these RNAs are the U family (for uridine-rich) of small nuclear RNAs (snRNAs) that are relatively stable, evolutionarily conserved, and associated *in vivo* with at least a set of common polypeptides, forming small nuclear ribonucleoprotein complexes (snRNPs). The set of polypeptides differs from those polypeptides associated with pre-mRNA, tRNA, or 5S RNA (2–4). On the basis of sequence complementarity to RNA sequences thought to be important for splicing, interest has focused on the possible role of these snRNPs in mediating RNA processing through RNA–RNA interactions (5–7); this hypothesis has received experimental support based on a nuclear splicing system (8).

Progress on analyzing the composition and possible function of the U family of snRNPs has come from the discovery that snRNPs are often the targets of the autoimmune disorder systemic lupus erythematosus (SLE) and that certain patient sera are sufficiently monospecific (toward snRNP components) to serve as molecular probes (2, 9–14). One serum known as anti-Sm immunoprecipitates snRNPs containing either U1, U2, U4, U5, or U6 snRNPs, whereas anti-RNP antibodies immunoprecipitate only those snRNP complexes containing U1 (2).

There are differing reports as to the antigens for these autoimmune antibodies and as to the polypeptides present on the snRNP complexes (2, 12–17). Using characterized autoimmune sera, we defined the polypeptide components of human snRNPs of the U family. We distinguished between the components of U1 snRNPs and those of the other category of snRNPs and also identified one *in vivo* modification unique to a U1-associated polypeptide.

MATERIALS AND METHODS

Cell Growth and Subcellular Fractionation. HeLa cells were grown at 37°C on SMEM (GIBCO) in suspension culture and labeled at 2–5 × 10^5 cells per ml. For testing sera, cells were labeled for 24 hr with [^{32}P]phosphate (2 mCi per 10^7 cells; 1 Ci = 3.7 × 10^{10} Bq). In experiments looking at polypeptide composition, cells were labeled for 4 hr or 24 hr with [^{35}S]methionine (5 mCi per 10^8 cells), [^{3}H]leucine (2 mCi per 10^8 cells), ^{75}Se-labeled methionine (^{75}Se-methionine; 0.5 mCi per 10^8 cells), or [^{32}P]phosphate (20 mCi per 10^8 cells).

Nuclei were prepared from quick-chilled cells essentially as described by Beyer *et al.* (3), with the addition of phenylmethylsulfonyl fluoride and aprotinin (Sigma) to inhibit proteolysis, and phenylmethysulfonyl fluoride to inhibit nuclease action. Washed cells were suspended in 5 ml of homogenate buffer (0.1 M NaCl/1 mM $MgCl_2$/10 mM Tris, pH 7.5) with 0.25% Nonidet P-40 and were lysed with a Dounce homogenizer, pestle A. The homogenate was diluted 1:1 with 1.8 M sucrose (in homogenate buffer) and centrifuged 15 min at 10,000 rpm in a Sorvall HB4 rotor.

Nuclei (free from cytoplasmic contamination as judged by phase microscopy) prepared from about 1 × 10^8 HeLa cells were resuspended in 1 ml of homogenate buffer at pH 8.5 and were broken by a 10-sec sonication. The clarified extract was used for immunoprecipitation.

Sera and Immunochemical Methods. Antisera were obtained from SLE patients as defined by the American Rheumatism Association (18). Sera for initial analysis were already defined as either anti-Sm or anti-RNP by the criteria of immunofluorescent cytological assay, hemagglutination, and diffusion (9, 11, 19). By using the protein transfer assay described below, these sera were tested further for reactivity against known potential antigens: DNA, RNA, histones, bulk chromosomal nonhistones, and ribosomal proteins. Sera that apparently were monospecific by the above criteria for either anti-Sm or anti-RNP antibodies were rescreened by us, using the criteria established by Lerner and Steitz (2): precipitation of specific sets of U-family snRNPs from extracts of mammalian nuclei. We then choose for the experiments sera of high titer exhibiting only the anti-RNP pattern—i.e., precipitating only U1 and no other U RNA—and high-titer sera exhibiting only the anti-Sm pattern—that is, precipitating the Sm-spectrum U RNAs (U1,

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Abbreviations: snRNA(s), small nuclear RNA(s); RNP, ribonucleoprotein; snRNP(s), small nuclear RNP(s); SLE, systemic lupus erythematosus.

U2, U4, U5, and U6) in proportion to their nuclear abundance.

Immunoprecipitations were done essentially as described by Kessler (20) with Formalin-fixed staphylococcal A cells (Bethesda Research Laboratories) that had been cleaned by heating in 3% $NaDodSO_4$/10% 2-mercaptoethanol just prior to use. Typically, 200 μl of RNP extract was incubated 20 min at 4°C with 20 μl of serum; 200 μl of a suspension of staphylococcal A cells was added, and the mixture was incubated 5 min. Protein A–antibody–antigen complexes were washed five times by centrifugation and resuspension in NET wash buffer: 150 mM NaCl/5 mM Tris·HCl, pH 7.4/0.5% Nonidet P-40. The final pellet was suspended in 2% $NaDodSO_4$ with either 0.5 M NaCl for RNA extraction or 10% 2-mercaptoethanol for protein extraction. RNA components of the antigen were eluted by extraction with phenol/chloroform/isoamyl alcohol, 50:45:5 (vol/vol), and precipitated with ethanol prior to gel electrophoresis. The RNA was analyzed by electrophoresis on 7 M urea/8% polyacrylamide denaturing gels (containing 30:1 acrylamide/bisacrylamide) in gel running buffer of 7 M urea/1 mM EDTA/0.1% $NaDodSO_4$/50 mM Tris borate, pH 8.3. Protein components of the antigen were isolated by boiling the $NaDodSO_4$/mercaptoethanol solution for 5 min, pelleting the staphylococcal A cells, removing the supernatant, and precipitating the proteins with ethanol.

Protein transfer and antibody blotting followed the methods of Towbin *et al.* (21) and Renart *et al.* (22). Proteins from gradient fractions or total nuclear extracts were precipitated with absolute ethanol and run on 12.5% $NaDodSO_4$ gels (23). The gel was electrophoretically blotted for 3 hr onto nitrocellulose paper (Schleicher & Schuell) prior to staining by using the gel running buffer without $NaDodSO_4$. After the paper was dried and potential background sites were blocked with 1% gelatin, the blot was incubated for 1 hr at 4°C with a 1:100 dilution of antibody into incubation buffer (10 mM Tris·HCl, pH 7.4/150 mM NaCl/0.25% gelatin/0.5% Nonidet P-40), washed, and incubated with ^{125}I-labeled protein A (4×10^6 cpm) for 1 hr at 22°C. After the blot was washed six times with incubation buffer, the transfer was dried and audioradiographed. Gels used for electrophoretic transfer were shrunk (after transfer) to their original size with 10–20% methanol/10% acetic acid and dried in a dialysis membrane for comparison to the audioradiograph of the transfer. Protein gels of ^3H- or ^{35}S-labeled proteins were soaked in EN^{3}HANCE (New England Nuclear) and dried prior to exposure. Molecular weights were assigned by comparison with six protein standards: lysozyme (14,300), myoglobin (17,200), chymotrypsinogen (25,700), aldolase (40,000), catalase (58,000), and bovine serum albumin (68,000).

Phosphoamino Acid Analysis. Proteins were examined for phosphoamino acid content essentially as described by Hunter and Sefton (24). Proteins from the immunoprecipitations were treated with pancreatic RNase and run on gels as described above. The bands were cut from the gel, and the protein was isolated (24). Hydrolysis was in 6 M HCl for 1 hr at 110°C. Phosphoamino acids were separated by electrophoresis on cellulose thin-layer plates at pH 1.9 in the first dimension and at pH 3.5 in the second dimension. Locations of internal standards of phosphorylated nucleotides (uridine and cytidine monophosphate) were determined in separate experiments with labeled nucleotides; the location of internal standards of phosphorylated amino acids (phosphoserine, phosphotyrosine, and phosphothreonine) were detected by staining with ninhydrin. The experimental spots were detected by autoradiography.

RESULTS

Identification of HeLa snRNP Polypeptides. To distinguish between polypeptides common to all snRNPs precipitated by anti-Sm antibodies and those specific to U1 or to the other complexes, we carried out sequential immunoprecipitations on the same nuclear extract. All U1 snRNPs were first precipitated with an excess of anti-RNP antibodies (and staphylococcal A cells), and the remaining snRNPs were subsequently precipitated with anti-Sm antibodies (Fig. 1). Anti-RNP serum precipitates polypeptides of 68,000, 26,000, 17,000, and 14,000 daltons (Fig. 1, lanes 1 and 2). Anti-Sm serum produces the same pattern when used without prior anti-RNP treatment; similar patterns are observed after 24 hr of labeling with [^3H]leucine, [^{35}S]methionine, or ^{75}Se-methionine (data not shown). For convenience we term these polypeptides P68, P26, P17, and P14, respectively. P26 is seen as a doublet, P27 and P26, on longer gels (data not shown, but see Fig. 2). After removal of all U1 snRNPs from solution (Fig. 1, lane 3), precipitation with anti-Sm brings down P26, P17, and P14. Thus, P68 is a U1-specific polypeptide, and the remaining four polypeptides appear to be shared in common among U1, U2, U4, U5, and U6 snRNPs.

Antigens of snRNPs Recognized by Anti-RNP and Anti-Sm Antibodies. An alternative identification of the polypeptides exploits the protein transfer method. Proteins from snRNP extracts were run out on $NaDodSO_4$ gels and electrophoretically transferred to nitrocellulose; the immobilized proteins were probed with antibody and iodinated protein A as described. Fig. 2 shows the reaction with anti-RNP (Fig. 2, lane A) and anti-Sm (Fig. 2, lane B). All six sera we tested that are apparently monospecific for the type anti-RNP (under current criteria as described) react uniquely with the 68,000-dalton polypeptide on nitrocellulose transfers. In contrast, six monospecific anti-Sm sera react with a doublet around 26,000 daltons and a single band at 17,000 daltons (Fig. 2). Although three antisera have been found previously to react only with the 26,000-dalton doublet (25), another antiserum we have tested reacts only with

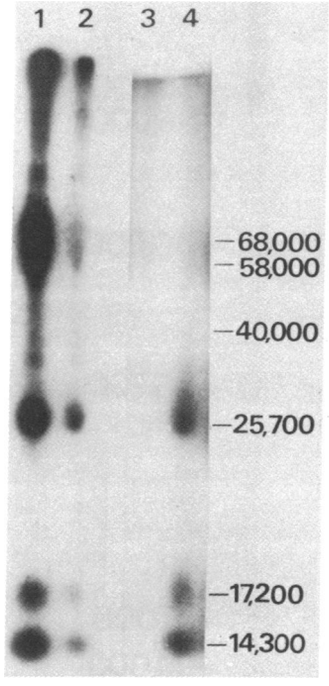

FIG. 1. Identification of U snRNP polypeptides. Proteins from immunoprecipitations of ^{75}Se-methionine-labeled cells were run on 12% $NaDodSO_4$ gel. Lanes: 1–3, sequential anti-RNP precipitations; 4, subsequent anti-Sm precipitation. The high molecular weight material observed also (i.e., >75,000 on this gel) precipitates in protein A control wells. Lanes 3 and 4 were exposed 50% longer than were lanes 1 and 2.

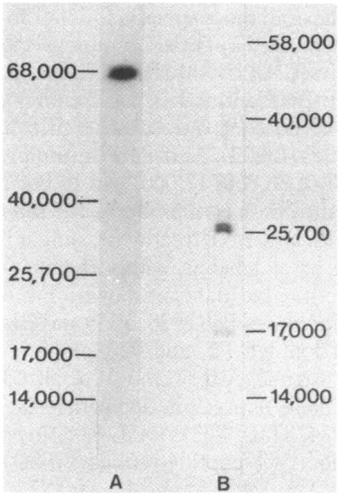

FIG. 2. Transfer radioimmunoassay of RNP and Sm antigens. Total unlabeled nuclear proteins from the sonic extract were run on 12% $NaDodSO_4$ gels and transferred to nitrocellulose. Patient SLE antibodies were allowed to react at a dilution of 1:100 in phosphate-buffered saline, and antibody binding was visualized with ^{125}I-labeled protein A. Lanes: A, anti-RNP antiserum; B, anti-Sm antiserum.

the 17,000-dalton polypeptide (data not shown).

Phosphorylation of HeLa snRNP Proteins. To look for modifications of the polypeptides, HeLa cells were labeled with ^{32}P for 24 hr, and snRNPs were recovered by immunoprecipitation of the sonic extract with either anti-RNP or a mixture of anti-RNP and anti-Sm antibodies. Protein extracts were treated with ribonuclease and run on $NaDodSO_4$ gels as described. Under these conditions, only the 68,000-dalton polypeptide was labeled (Fig. 3). To determine the modified residue, the 68,000-dalton species was cut from the gel, the protein was hydrolyzed, and the amino acids were run on two-dimensional thin-layer plates (Fig. 4). The only modified amino acid found was phosphoserine. No labeled nucleotide spots were found in the region of the gel of the 68,000-dalton protein.

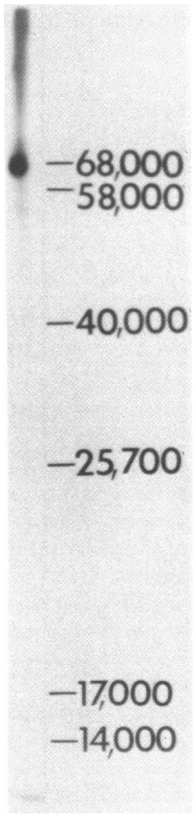

FIG. 3. Phosphorylated snRNP polypeptides. SnRNPs from [^{32}P]-phosphate-labeled cells were precipitated with a mixture of anti-Sm and anti-RNP antibodies. Proteins from the extract were treated with pancreatic ribonuclease and run on a 12% $NaDodSO_4$ gel.

DISCUSSION

On the basis of transfer-blotting experiments and precipitation of labeled polypeptides, we have identified a set of polypeptides common to the U family of RNPs defined by their reaction with anti-Sm, namely, U1, U2, U4, U5, and U6 snRNPs. These polypeptides are 14,000, 17,000, 26,000, and 27,000 daltons by our calibration; we term these P14, P17, P26, and P27. There may be polypeptides of lower abundance that we have not observed. Reliable detection or determination of polypeptides unique to the 90% less abundant U4, U5, or U6 snRNPs is not possible in immunoprecipitates of sonic extracts and would require prior purification of these complexes. P26 and P27 correspond to the B species, P17 corresponds to the D polypeptide, and P14 to the E polypeptide, originally identified for mouse cells by Lerner and Steitz (2). We have never seen polypeptides smaller that P14, although smaller polypeptides have been found previously (2, 12–17). It is possible, but unlikely, that these polypeptides are lost during washing of our immunoprecipitations. As long as the protease inhibitor aprotinin is included, we do not see appreciable amounts of material corresponding to two other polypeptides identified for mouse cells by Lerner and Steitz (2): the A polypeptide or the C polypeptide. However, these polypeptides appear to have been identified (in human cells) by others (12–14). It may be that the P68 is a precursor to the A and C polypeptides only in mouse cells, not in human cells, but structural similarities allow artifactually similar cleavages in human extracts. Because P17 is an antigen for anti-Sm based on the transfer-blotting experiments, it is probably not a breakdown product. P14 is consistently and abundantly present in the snRNP precipitate but it is not reactive with any anti-Sm serum we have tested. If it is a unique polypeptide, P14 must be buried or inaccessible within snRNPs *in vivo* and, thus, not be a target for the autoimmune response. However, the relationship of the smaller common polypeptides, P14 and P17, to the larger ones, P26 and P27, only will be rigorously established by analysis of partial peptide maps and amino acid composition.

We have now identified a U1-specific polypeptide at 68,000 daltons, P68, that appears to be the antigen for anti-RNP antibody, although a polypeptide of this size has not commonly been found. In transfer-blotting experiments with a mouse monoclonal anti-RNP antibody developed by Billings *et al.* (26), we find that this monoclonal antibody reacts in our hands only with the 68,000-dalton polypeptide and also provides a similar immunoprecipitation pattern to what we find with the polyclonal patient sera (data not shown). Takano *et al.* (12) and Hoch's laboratory (14, 26) also have identified the 68,000-dalton polypeptide as an anti-RNP antigen, although they saw smaller polypeptides (notably one corresponding to the A polypeptide) reactive with the antibody as well. We believe that the low molecular mass proteins are degradation products of the 68,000-dalton protein; in the absence of aprotinin, we see U1-specific polypeptides of 34,000 daltons and a doublet at 20,000 and 22,000 daltons (unpublished observations), consistent with antigens or polypeptides found by others (2, 12–15, 26). Protein transfer and blotting of the replica represents an experiment with less

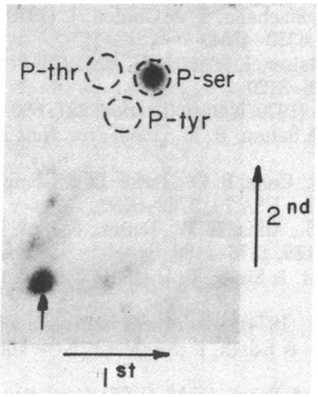

FIG. 4. Phosphoamino acid analysis of P68. An autoradiogram of the thin-layer chromatography plate for the two-dimensional phosphoamino acid analysis of hydrolyzed P68 is shown. The origin was in the lower left (shown by arrow) with the first dimension at pH 1.9 from left to right and the second dimension at pH 3.5 from bottom to top. Location of phosphoamino acid standards is shown by circles. No spots for phosphonucleotides, phosphotyrosine, or phosphothreonine were seen in comparison with internal standards.

chance for proteolysis, compared to more extensive handling for immunoprecipitation of extracts with antisera. Thus, this specificity on the blot strongly supports the argument that there is only one U1-specific polypeptide.

Work in the autoimmune field in rheumatology has focused on identifying single antigens for these autoimmune diseases; it now appears that more than one antigen is reactive against anti-Sm from our work and work in the field; this would be expected from such a complex macromolecular species as the U family of snRNPs. This finding explains the broad range of immunoreactions seen in a variety of laboratories because different antisera are capable of reacting with different polypeptides. In addition, the complexes are clearly very sensitive to autolysis; this probably explains the early reports that suggested that all snRNP polypeptides were about 13,000 daltons in size (e.g., refs. 12–14, 17). Cryptic specificity such as anti-histone antibodies or anti-high mobility group antibodies also may have complicated studies that use conventional means of identification of antigens. The use of complex polyclonal antibodies from patient antisera requires testing by both the RNA precipitation method and the protein transfer method of radioimmunoassay; the latter is the most sensitive method for detecting cryptic contamination. On the other hand, the smaller polypeptides that result from autolysis are so well defined as to suggest that the cleavage indicates structural domains of the molecules and that these observations provide a first step toward a defined attack using proteases (and nucleases) to define structural domains of snRNP complexes.

We have found previously that the Sm antigenic polypeptides are conserved in molecular weight, i.e., anti-Sm antibodies react with 26,000-dalton and 18,000-dalton polypeptides in *Drosophila* extracts, which correspond in HeLa extracts to the 26,000-dalton doublet and the 17,000-dalton polypeptide, respectively (25). On replica transfers, the *Drosophila* 26,000-dalton band was also a doublet (unpublished observations); this suggests that P27 and P26 are separate polypeptides, not modified forms of each other. Another feature of similarity between HeLa and *Drosophila* snRNPs concerns the P14 polypeptide because we find it in anti-Sm immunoprecipitations of *Drosophila* extracts as well (unpublished observation). Thus, not only are the U snRNA sequences conserved (25, 27), but also the protein number and size of common snRNP polypeptides are conserved.

Early studies suggested that the antigen for anti-Sm antibodies is trypsin sensitive whereas the antigen for anti-RNP is both ribonuclease and trypsin sensitive (9–11). White *et al.* (13) have shown that the Sm antigen becomes ribonuclease sensitive when it is more thoroughly purified, as might be expected for an snRNP complex. It is not surprising that we and others (12, 14, 26) have found that the 68,000-dalton polypeptide is an antigen for RNP despite its protein nature because it seems likely that the ribonuclease sensitivity is a relative one that depends on the method of assay. Rather than a heterologous antigenic determinant containing both RNA and protein, it seems likely that the native form of the protein is more reactive with the antibody in the presence of RNA and that after ribonuclease digestion the reaction is greatly diminished. The reaction in our hands is much weaker with the immobilized P68 antigen than it is in solution because we cannot dilute anti-RNP antisera more than 1:200 and still get a significant reaction on a blot even though the sera can be diluted 10,000 times or more and still bind to nuclei (unpublished). We first suspected the 68,000-dalton polypeptide might contain RNA and that this would explain its reactivity with anti-RNP antisera after transfer from the protein gel. However, the polypeptide fails to label when cells are labeled with nucleotides and no nucleotides are seen on the two-dimensional phosphoamino acid analysis, even when RNase pretreatment is omitted (unpublished data).

Protein phosphorylation is a post-translational modification that can modulate protein function and can regulate the ability of proteins to interact with other proteins (e.g., see refs. 28–30). We find P68 contains phosphoserine. There may be other low-level phosphorylated amino acids not detected in our assay of phosphorylated proteins. However, it is intriguing that the only phosphorylated residues occur on the U1-specific polypeptide. We suspect that there is more than one phosphoserine residue because, in ^{32}P-labeled cultures, P68 reaches 1–5% of the extent of labeling of U1. In addition, we find the putative P68 breakdown products at 34,000 daltons (A polypeptide) and 20,000 and 22,000 daltons (the C doublet polypeptides) all contain phosphoserine (data not shown). It is obvious but striking that phosphoserine residues will not bind RNA and their repulsion might be important for P68–RNA interactions.

Note Added in Proof. Hinterberger *et al.* (31) have fractionated mouse snRNPs and found enrichment in snRNPs containing U1 for polypeptides of 68,000, 34,000, and 22,000 daltons, consistent with our observations reported above. Kinlaw *et al.* (32) have observed a methionine-deficient, 27,000-dalton polypeptide unique to snRNPs containing U2.

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